Anti-PD-1 Antibody Therapy Potently Enhances the Eradication of Established Tumors By Gene-Modified T Cells

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Abstract

Purpose: To determine the antitumor efficacy and toxicity of a novel combination approach involving adoptive T-cell immunotherapy using chimeric antigen receptor (CAR) T cells with an immunomodulatory reagent for blocking immunosuppression.

Experimental Design: We examined whether administration of a PD-1 blocking antibody could increase the therapeutic activity of CAR T cells against two different Her-2+ tumors. The use of a self-antigen mouse model enabled investigation into the efficacy, mechanism, and toxicity of this combination approach.

Results: In this study, we first showed a significant increase in the level of PD-1 expressed on transduced anti-Her-2 CD8+ T cells following antigen-specific stimulation with PD-L1+ tumor cells and that markers of activation and proliferation were increased in anti-Her-2 T cells in the presence of anti-PD-1 antibody. In adoptive transfer studies in Her-2 transgenic recipient mice, we showed a significant improvement in growth inhibition of two different Her-2+ tumors treated with anti-Her-2 T cells in combination with anti-PD-1 antibody. The therapeutic effects observed correlated with increased function of anti-Her-2 T cells following PD-1 blockade. Strikingly, a significant decrease in the percentage of Gr1+CD11b+ myeloid-derived suppressor cells (MDSC) was observed in the tumor microenvironment of mice treated with the combination therapy. Importantly, increased antitumor effects were not associated with any autoimmune pathology in normal tissue expressing Her-2 antigen.

Conclusion: This study shows that specifically blocking PD-1 immunosuppression can potently enhance CAR T-cell therapy that has significant implications for potentially improving therapeutic outcomes of this approach in patients with cancer. Clin Cancer Res; 19(20); 1–11. ©2013 AACR.

Introduction

Adoptive immunotherapy using gene-modified T cells expressing antigen-specific chimeric antigen receptors (CAR) is a promising approach for the treatment of cancer (1–3). Recent clinical trials using CARs that target cell surface tumor-associated antigens such as CD20 for the treatment of non-Hodgkin lymphoma (4), GD2 in neuroblastoma (5), and CD19 in chronic lymphocytic leukemia (6, 7) have all displayed encouraging results to date resulting in objective antitumor responses in a proportion of patients. These trials clearly illustrate the broad range of tumors that can be targeted by CAR T cells in a HLA-independent manner (8).

Despite encouraging results in preclinical models and in patients, the existence of a number of different immunosuppressive pathways can restrict the full potential of adoptive T-cell therapy. This includes increased expression of inhibitory immune receptors such as T-cell membrane protein-3 (TIM-3), cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), and/or programmed death-1 (PD-1) on T cells following T-cell activation, which can limit the duration and strength of the adaptive immune response (9). However, the recent development of checkpoint inhibitors such as ipilimumab targeting CTLA-4 (10), provide diverse opportunities to enhance antitumor immunity with the potential to produce durable clinical responses (9).

The PD-1 pathway has emerged as another promising target for cancer therapy. PD-1 binds to two known ligands; PD-L1, the predominant mediator of immunosuppression which is upregulated on many different tumor types and
Translational Relevance
In this study, we show that blockade of the PD-1 immunosuppressive pathway using an anti-PD-1 antibody can significantly enhance the antitumor efficacy of genetically modified T cells expressing a chimeric antigen receptor (CAR). This combination therapy was shown to significantly inhibit tumor growth in two different mouse models leading to eradication of disease in a proportion of mice. Both of these approaches have been used singly in the clinic showing good safety profiles where objective and complete responses have been reported against various cancer types. However, many patients do not respond to either treatment alone. The current study shows that combining these two modalities can dramatically increase antitumor effects against established disease. Furthermore, we show that the increased effects from combination therapy did not cause pathology in mice and that therapeutic responses strongly correlated with a decrease in MDSCs.

PD-L2 which has restricted expression on macrophages and dendritic cells (11). Engagement of the PD-1/PD-L1 pathway results in the phosphorylation of tyrosine-based motifs in the cytoplasmic tail of the PD-1 inhibitory receptor, which promotes the recruitment of SHP2 phosphatase leading to dephosphorylation of PI3K. As a result, inhibition of PI3K effects downstream activation of Akt kinase reducing T-cell activation, proliferation, and survival (12, 13). Recent trials using a fully human IgG4 PD-1 monoclonal antibody (mAb; BMS-936558) have reported durable clinical responses in patients with advanced melanoma, non–small cell lung, renal cell, as well as hematologic malignancies (11, 13–15). Furthermore, a significant correlation between level of PD-L1 expression on tumor cells and objective responses was reported (14). Another clinical trial used a human IgG4 anti-PD-L1 mAb which showed promising efficacy against a range of cancers with objective responses reported in a proportion of patients (16). Given the early success of these checkpoint inhibitors in enhancing T-cell immunity, it opens the possibility that these reagents could be used to enhance adoptive T-cell strategies.

Hence, in this study, we hypothesized that CAR T-cell therapy in combination with PD-1 blockade may overcome PD-L1+ tumor immunosuppression, thereby leading to improved therapeutic efficacy. Herein, we used self-antigen Her2 transgenic mice and used two different Her-2+ tumor targets to examine both antitumor efficacy and safety of CAR T cells and anti-PD-1 antibody in a combined immunotherapeutic approach.

Materials and Methods

Cell culture, antibodies, and mice
The C57BL/6 mouse 24JK sarcoma cell line was kindly provided by Dr. Patrick Hwu (NIH, Bethesda, MD) and maintained at 37°C in 5% CO2 in RPMI-1640 media supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mmol/L glutamine, 1 mmol/L sodium pyruvate, 0.1 mmol/L nonessential amino acids, 100 U/mL penicillin, and 100 μg/mL streptomycin (Life technologies). The B6 mouse e0771 (LMC variant) breast cancer line was kindly provided by Prof. Robin Anderson (Peter MacCallum Cancer Centre, Victoria, Australia) and maintained at 37°C in 10% CO2 in Dulbecco’s modified Eagle medium (DMEM), supplemented as above with 5% FCS. These lines were retrovirally transduced with the human Her-2 antigen (24JK-Her-2/e0771-Her-2) as described previously (17). The anti-PD-1 (clone RMP1-14, IgG, Protein G purified, endotoxin-free) and isotype (clone UB8-1B9) antibodies were derived from Bio-XCell and in-house, respectively. C57BL/6 human Her-2 transgenic mice and congenic Thy1.1+ Her-2 mice were bred at the Peter MacCallum Cancer Centre and used for experimentation at 6 to 16 weeks of age. All animal experimentation was approved in advance by the Peter MacCallum Cancer Centre Animal Experimentation Ethics Committee.

Generation of gene-modified T cells
The GP+E86 packaging line expressing the CAR containing the extracellular scFv-anti-Her-2 human monoclonal antibody (mAb) region fused to the transmembrane and intracellular costimulatory domain CD28 and intracellular TCR-ζ domain was generated as previously described (18, 19). For transduction, splenocytes from C57BL/6 Her-2 or Thy1.1+ Her-2 mice transgenic mice were gene modified as previously described (20). Following transduction, anti-Her-2 T cells were cultured in RPMI-1640, supplemented with 100 U/mL of IL-2 and 2 ng/mL of IL-7, and used for in vitro and in vivo experiments.

Flow cytometry
Transduction efficiency of T cells expressing the chimeric anti-Her-2 receptor was determined by direct immunofluorescence with a myc-tag Alexa Fluor 488–conjugated immunoglobulin (Cell Signaling Technology). Background immunofluorescence was assessed using an IgG2a Alexa Fluor 488–conjugated mouse isotype immunoglobulin (Invitrogen). Phenotypic characterization of transduced cells was determined using direct staining with myc-Tag (clone 9B11; Cell Signaling), TCRβ (clone H57-597; Jomar Diagnostics), CD4 (clone RM4-5; BD Pharmingen), CD8 (clone 53-6.7; BD Pharmingen), and PD-1 (clone J43; Jomar Diagnostics) cell surface markers. Intracellular staining for IFN-γ (clone XMG1.2; BD Pharmingen), and Ki67 (clone B56; BD Bioscience) was carried out using a Cytofix/Cytoperm, Fixation/Permeabilization Solution Kit in accordance with manufacturers’ instructions (BD Bioscience). Tumors were examined for PD-L1 cell surface expression by double staining with biotinylated PD-L1 (clone MH5; Jomar Diagnostics) and Streptavidin-APC (Jomar Diagnostics). For T-cell function and localization studies, tumors and blood were removed from mice at day 2 and 8 posttreatment.
(n = 6–8/group) and processed as previously described (21). Cells were stained with a cocktail of antibodies for identifying presence of various immune cell populations (TCRβ, Thy1.1, CD4, CD25, FR4, CD11b, Gr-1, F4/80) and intracellular IFN-γ. Gr1+ cells were examined for PD-L1 and PD-1 cell surface expression by double staining with biotinylated PD-L1 (clone MHI5; Jomar Diagnostics) and Streptavidin-Pe-Cy7 (Jomar Diagnostics) or phycoerythrin (PE)-conjugated PD-1 (clone J43; Jomar Diagnostics). Samples were incubated for 25 minutes at 4°C and analyzed by flow cytometry (LSR-II, BD Biosciences, North Ryde, New South Wales, Australia).

Adoptive cell therapy experiments
C57BL/6 human Her-2 transgenic mice (n = 5–8) were injected subcutaneously with 1 × 106 24JK-Her-2 cells or orthotopically with 5 × 105 e0771-Her-2 breast carcinoma cells into the fourth mammary fat pad. Mice were then preconditioned (5 Gy) on Day 7 posttumor injection, before transfer of scFv-anti-Her-2–transduced T cells or control LXSN–transduced T cells alone (1 × 107/dose on days 7 and 8) or T cells in combination with either anti-PD-1 or isotype antibody (250 μg/injection on days 7, 11, and 15). Control groups were left untreated. Tumor growth in mice was monitored by twice weekly measurements using electronic calipers (length × height) and the area of the tumor determined for each mouse. The mean tumor area ± SEM was recorded for each treatment group. In some tumor experiments, recipient mice received transduced donor T cells preconditioned (5 Gy) on Day 7 posttumor injection, before transfer of scFv-anti-Her-2–transduced T cells or control LXSN–transduced T cells alone (1 × 107/dose on days 7 and 8) or T cells in combination with either anti-PD-1 or isotype antibody (250 μg/injection on days 7, 11, and 15). Control groups were left untreated. Tumor growth in mice was monitored by twice weekly measurements using electronic calipers (length × height) and the area of the tumor determined for each mouse. The mean tumor area ± SEM was recorded for each treatment group. In some experiments, recipient mice received transduced donor T cells when cocultured with e0771-Her-2 tumor targets. All of these tumor lines displayed high level expression of the PD-L1 surface marker (Supplementary Fig. S2). Further analysis revealed that increased PD-1 expression on anti-Her-2 T cells following antigen stimulation
Activation of T cells through TCR recognition of MHC/peptide leads to enhanced PD-1 expression which can reduce T-cell function and ultimately lead to T-cell exhaustion (13). To determine whether stimulation of T cells through the chimeric antigen receptor similarly leads to increased expression of PD-1, we first evaluated the expression of the PD-1 ligand (PD-L1) on Her-2+ (24JK-Her-2, e0771-Her-2) and parental (24JK, e0771) tumor targets. All of these tumor lines displayed high level expression of the PD-L1 surface marker (Supplementary Fig. 1A–D). A similar finding was also shown when T cells were cocultured with 24JK and 24JK-Her-2. Interestingly, we observed a significantly increased level of PD-1 receptor expression on the total transduced (Tag+) T-cell population when cocultured with Her-2+ target cells (11.86 ± 3.14% SEM) compared with stimulation with 24JK parental tumor cells (6.45 ± 0.45% SEM; Fig. 2A). A similar finding was also shown when T cells were cocultured with e0771-Her2 tumor targets (Supplementary Fig. S2). Further analysis revealed that increased PD-1 expression was only observed on CD8+ T cells when cocultured with 24JK-Her-2 target cells (9.23 ± 3.77% SEM), compared to stimulation with parental tumor cells (3.40 ± 0.58% SEM) or in media alone (5.16 ± 0.84% SEM; Fig. 2A). In contrast, there was no significant increase in PD-1 receptor expression on transduced CD4+ T cells after antigen stimulation (3.21 ± 0.38% SEM; Fig. 2A).

PD-1 blockade enhances the proliferative and functional capacity of anti-Her-2 T cells in vitro
The functional capacity of anti-Her-2 T cells cocultured with 24JK cells expressing human Her-2 antigen (24JK-Her-2) in combination with anti-PD-1 or isotype antibody was determined by staining for intracellular markers of activation by flow cytometry. Following overnight stimulation, we found that blockade of PD-1 significantly enhanced the intracellular expression of the proliferation marker Ki-67, as well as IFN-γ and granzyme B, compared with
isotype control antibody or transduced T cells cocultured with parental 24JK tumor cells (Fig. 2B–D). These results suggest that blocking PD-1 immunosuppression can increase important functional parameters in CAR T cells following stimulation through the CAR.

Adoptive transfer of anti-Her-2 T cells in combination with PD-1 blockade enhances regression of established tumor

We have previously shown that adoptive transfer of T cells gene-modified with the scFv-anti-Her-2 receptor could specifically mediate regression of 24JK-Her-2 experimental lung metastasis in Her-2 transgenic mice, although this therapy was less effective against established subcutaneous disease (20). Given that PD-1 expression was upregulated on CAR T cells following antigen stimulation in vitro, this raised the possibility that anti-PD-1 blockade may combine to enhance antitumor effects mediated by adoptively transferred anti-Her-2 T cells in vivo. In this experiment, we compared the tumor growth and survival of Her-2 transgenic mice bearing day 7 established 24JK-Her-2 subcutaneous tumors (~20–30 mm³) treated with anti-Her-2 or control LXSN–transduced T cells with or without anti-PD-1 antibody. Treatment of mice with anti-Her-2 T cells and anti-PD-1 antibody led to the strongest growth inhibition of 24JK-Her-2 tumors (Fig. 3A) and long-term survival (~63%) of mice (Fig. 3B) that was statistically significant to mice treated with anti-Her-2 T cells alone or in combination with a control isotype antibody (Fig. 3A and B). Mice treated with anti-PD-1 alone or control T cells with anti-PD-1 had no effect on tumor growth or survival of mice (Fig. 3A and B). To show the broad use of this combined therapy, we showed that anti-Her-2 T cell and anti-PD-1 antibody treatment significantly reduced the growth of day 7 established e0771-Her-2 breast carcinoma tumors injected orthotopically into Her-2 transgenic mice compared with anti-Her-2 T cells alone or in combination with isotype control antibody (Fig. 3C). Collectively, these data show for the first time that the combination of adoptive immunotherapy using gene-modified CART cells with PD-1 blockade could significantly enhance antitumor effects against established cancer in an immune competent self-antigen setting.

Antitumor effects correlate with increased function of anti-Her-2 T cells but not localization at the tumor site following combination therapy

To determine whether the enhanced antitumor effects observed following combined CAR T cell and anti-PD-1 antibody therapy correlated with increased function and localization of anti-Her-2 T cells at the tumor site, mice were
challenged with 24JK-Her-2 tumor and irradiated before receiving anti-Her-2 Thy1.1 T cells with either anti-PD-1 or control isotype antibody. In this experiment and consistent with our in vitro data, we observed significantly increased expression of intracellular IFN-γ in adoptively transferred Thy1.1+ T cells at day 2 following anti-PD-1 blockade compared with isotype control-treated mice (Fig. 4A). However, although Thy1.1+ donor T cells were detectable in both the tumor (Fig. 4B and C) and blood (Fig. 4D) of mice that received anti-Her-2 T-cells, there was no difference in the percentage of Thy1.1+ T-cells between groups of mice that received anti-PD-1 compared with isotype control antibody (Fig. 4B–D). Given that there was no impact of PD-1 blockade on the percentage of adoptively transferred T cells at the tumor site, our data raised the possibility that PD-1 blockade on the percentage of adoptively transferred T cells or anti-PD-1 mAb. Interestingly, we also observed a

Antitumor effects correlate with a decrease in myeloid derived suppressor cells at the tumor site following combination therapy

Given that PD-1 is known to be expressed on immunosuppressive cells including T regulatory cells (Treg; ref. 22) and myeloid-derived suppressor cells (23, 24), we next examined the effect of combined anti-Her-2 CAR T cell and anti-PD-1 therapy on these different cell populations. In this experiment, tumor and blood were taken at day 1 and 8 posttreatment from control mice and mice that received anti-Her-2 T cells with or without anti-PD-1 antibody and analyzed by flow cytometry. Although no significant difference in the percentage of CD4+ T cells, Tregs, or Gr-1+ cells was observed at the tumor site at day 1 posttherapy between treatment groups (Fig. 5A), we strikingly found a significant reduction in percentage of CD11b+ Gr-1+ cells (MDSCs) in tumors of mice treated with CAR T cells and anti-PD-1 antibody, compared with nontreated controls at day 8 posttherapy (Fig. 5B). In contrast, there was no significant difference between mice treated with anti-Her-2 T cells and control isotype antibody and nontreated mice at this time point. Our data suggest that the addition of anti-PD-1 mAb with CAR T cells has contributed to the significant decrease in percentage of MDSCs given the decrease was greater than that observed with CAR T cells combined with isotype control antibody. Nevertheless, our experiments do not formally distinguish the relative contribution of either CAR T cells or anti-PD-1 mAb.
significant decrease in percentage of CD11b+ Gr-1+ cells in the blood of mice that received combination therapy compared with nontreated mice at day 1 posttherapy (Fig. 5C) and a moderate decrease in the blood at day 8 (Fig. 5D). These data fit well with the fact that these Gr-1+ cells isolated from 24JK-Her-2 tumors were found to express both PD-1 (albeit at low levels) and PD-L1 (Supplementary Fig. S3). In contrast with the effects on MDSCs, there was no additional modulation of Treg cells at the tumor site following combination therapy at day 8 (Fig. 5B). Notably though, the percentage of total CD4 T cells and Treg cells in the tumor at day 8 posttherapy were similarly decreased in both anti-Her-2–treated mice with or without anti-PD-1 antibody compared with untreated control mice (Fig. 5B). This correlated with a concomitant increase in number of Thy1.1+ donor T cells in the tumor of these mice (Fig. 4C). Collectively, our data show a strong correlation between decreased numbers of MDSCs and increased therapeutic responses mediated by combined CAR T cell and anti-PD-1 antibody therapy.

Adoptive transfer of CAR T cells with PD-1 blockade does not cause autoimmunity in mice

The human Her-2 antigen is reportedly expressed as a self-antigen in parts of the brain (cerebellum) and mammary tissue of Her-2 transgenic mice (20, 25). We have previously
shown that adoptive transfer of anti-Her-2 T cells alone did not induce pathology to normal tissue expressing Her-2 antigen in these mice (20). However, given the significantly increased antitumor effects observed in Her-2 transgenic mice following combination therapy, it was important to examine potential toxicity to Her-2+ normal tissue in this model. In this experiment, we conducted H&E staining of brain and mammary sections from Her-2 transgenic mice that were irradiated and challenged with Her-2+ tumor and received anti-Her-2 T cells in combination with or without anti-PD-1, as previously described. Brain and mammary tissues were then thoroughly assessed for any potential signals of T-cell–mediated pathology such as inflammation caused by immune infiltration. Interestingly, all mice treated with T cells expressing the anti-Her-2 receptor either alone or in combination with anti-PD-1 antibody showed no tissue damage, as shown by H&E staining of mammary or brain sections from representative mice at day 8 posttreatment (Fig. 6). The morphologic appearance of sections from these tissues was comparable with sections from nontreated mice (Fig. 6). Thus, the transfer of CAR T cells with PD-1 blockade did not induce any significant autoimmunity after early treatment indicating this combination therapy to be both effective and safe.

**Discussion**

Adoptive immunotherapy using gene-modified T cells has emerged as a promising approach for the treatment of a broad range of cancers (8). However, the presence of an immunosuppressive microenvironment induced by tumors and host regulatory cells can limit the full potential of adoptive T-cell immunotherapy. One such regulatory pathway includes PD-1/PD-L1 which serves to act as a negative feedback loop to switch off adaptive immunity after the initial immune response (13). Recently, the use of checkpoint inhibitors targeting the PD-1/PD-L1 pathway has shown remarkable antitumor responses in patients with advanced melanoma and against other cancers with durable clinical responses reported in a proportion of cases (9, 14–16). Given that PD-1 receptor expression is increased on activated T cells following engagement of PD-L1 ligand on target cells (26), we hypothesized that blocking this pathway may further enhance the antitumor activity of gene-modified T cells. Indeed, we first showed that expression of the PD-1 receptor was significantly increased on CAR T cells

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**Figure 4. Antitumor effects correlate with increased function of anti-Her-2 T cells but not localization at the tumor site following combination therapy.** The frequency of anti-Her-2 Thy1.1+ T cells in the tumor expressing intracellular IFN-γ (A), and total (black bars, TCRβ+) and anti-Her-2 Thy1.1+ (white bars, TCRβ+) Thy1.1+ T-cell numbers in the tumor (B and C) and blood (D) of irradiated 24JK-Her-2 tumor-bearing mice that were treated with or without anti-PD-1 or isotype control antibody and IL-2 was investigated by flow cytometry at day 2 and 8 posttherapy. A significant increase in the percentage of IFN-γ–expressing Thy1.1+ donor T cells in the tumor of mice following anti-PD-1 blockade was observed compared with isotype control-treated mice (*, P ≤ 0.05). A significant increase in the percentage of Thy1.1+ donor T cells in the tumor and blood of mice that received anti-Her-2 T cells was also observed compared with nontreated mice. There was no significant difference in the percentage of donor T cells following administration of anti-PD-1 or isotype antibody (†, P < 0.05; ††, P < 0.01; †††, P < 0.001). Data shown are the mean percentage ± SEM of total viable GFP− cells (nontumor cells) from 6 to 8 mice analyzed.
following coculture with PD-L1+ Her-2+ expressing tumor targets and that PD-1 blockade enhanced functional parameters of CAR T cells in vitro and in vivo. In adoptive transfer experiments, we showed that combined CART-cell therapy with anti-PD-1 antibody significantly increased the growth inhibition of two different Her-2+ tumors and led to enhanced survival of transgenic Her-2 recipient mice. Interestingly, we found that increased antitumor responses mediated by this combined therapy correlated well with a significant decrease in numbers of CD11b+ Gr-1+ myeloid-derived suppressor cells (MDSCs) but not Treg cells at the tumor site compared with nontreated control mice. Importantly, CART-cell treatment combined with anti-PD-1 antibody administration was well tolerated causing no signs of autoimmunity in recipient mice. Overall, this is the first study to show that blocking a major immunosuppressive pathway such as PD-1 can significantly enhance adoptive immunotherapy using gene-modified T cells. Although the question of whether PD-L1 ligand expressed on the tumor cells was critical for the therapeutic effects observed was not determined in this study, a significant correlation between the level of PD-L1 expression on tumor cells and objective responses in patients has been previously reported (14).

We have previously shown that CART T-cell therapy alone could elicit significant antitumor effects on the growth of established 24JK-Her-2+ lung metastases leading to long-term survival without causing any signs of pathology in vivo (20). However, in this current study, CART T-cell therapy alone was not as effective against established subcutaneous disease. Nevertheless, we were able to show in our in vivo experiments striking growth inhibition of both 24JK-Her-2+ sarcoma cells and E0771-Her-2+ breast carcinoma cells when inoculated subcutaneously following CART-cell therapy in combination with PD-1 blockade compared with mice treated with CART cells and isotype control antibody. We found both in vitro and in vivo that blocking PD-1 significantly enhanced the function of CART T cells leading to a more lethal hit against the tumor. Unlike other studies that used MHC class I restricted T cells (26, 27), we found no increase in the percentage of adoptively transferred Thy.1+ T cells in the blood or tumor site following combined therapy compared with control treated mice. Future studies using other qualitative approaches such as luciferase transduced T cells would enable the kinetics of T-cell infiltration to be better defined (27).

An interesting aspect of the current study was that the strong antitumor effects observed with CART cells...
combined with anti-PD-1 antibody correlated with a significant decrease in the percentage of Gr-1$^+$ CD11b$^+$ MDSCs at the tumor site. Given that the percentage of Gr-1$^+$ CD11b$^+$ cells expressing PD-1 was found to be low and that anti-PD-1 has been reported to be a blocking antibody rather than a depleting antibody (28, 29) we believe that the increased antitumor effects observed using anti-PD-1 in our tumor models were most likely due to an indirect effect on the MDSC population. Our results are consistent with another study showing a reduction of myeloid cells in tumors following anti-PD-1 therapy (30). Previous studies using anti-PD-1 alone or in combination with anti-CTLA-4 have shown a reduction in the suppressive activity of MDSCs through inhibition of arginase I (24). Other studies have shown that MDSCs can suppress antitumor responses through production of nitric oxide synthase (31), reactive oxygen species (32), and cytokines such as TGF-$\beta$ and IL-10 (33, 34). It remains to be determined in future studies whether the increased antitumor effects of CAR T-cell therapy in combination with PD-1 blockade was through modulation of the immunosuppressive effects of MDSCs. In contrast with the effects on MDSC numbers, we observed no additional effect on percentage of Treg cells in the blood or tumor site following combined CAR T-cell and anti-PD-1 treatment. The decreased percentage of Treg cells and concomitant increase in Thy1.1$^+$ T cells in Her-2 recipient mice was most likely due to the effects of irradiation which has been reported to decrease host immunosuppressive cell populations enabling expansion of transferred T cells (35). Although PD-1 blockade alone has previously been reported to decrease Treg numbers (30), our findings are consistent with another report showing that anti-PD-1 antibody treatment did not modulate Treg cell numbers following adoptive immunotherapy (27). It is important to note that these studies do not preclude an effect of PD-1 blockade on the function of Treg cells within the tumor microenvironment.

Another important aspect of our work was that the use of self-antigen Her-2 transgenic mice allowed us to assess whether CART-cell therapy combined with anti-PD-1 caused any pathology to normal tissue expressing the Her-2 antigen. Although we have previously reported no toxicity of anti-Her-2 CAR T cells alone in this model (20), it remained possible that significant increases in therapeutic activity using anti-PD-1 could have concomitantly led to autoimmunity. In this current study, detailed immunohistochemical analysis...
revealed no damage to either Her-2+ mammary or brain tissue following combined therapy. This may have been due to the lower levels of Her-2 antigen expressed on normal tissue compared with 24JK-Her-2 tumor cells as we have reported previously (20) or due to insensitivity of the Her-2 transgenic mouse model for detecting such autoimmune events. Nevertheless, our data are encouraging for moving towards testing this combined approach in the clinic. A number of recent clinical trials involving adoptive transfer of CAR T cells alone has shown promising results in patients with chronic lymphoid leukaemia and neuroblastoma with reports of long-term remission in some cases (5, 7). Nevertheless, other trials using CAR T cells have reported on target toxicity following treatment (36–38). Similarly, recent clinical trials using antibodies targeting the PD-1 receptor and its ligand PD-L1 have reported durable responses against various solid cancers (14–16). In these trials, treatment was generally well tolerated although some drug-associated adverse events and pneumonitis was reported in a small percentage of patients (14). Given these reported toxicities in some patients, it will be important in future studies to further optimize dose and timing regimens of CAR T cells with anti-PD-1 antibody in self-antigen mouse models before phase I testing.

In conclusion, our studies have shown for the first time that administration of an anti-PD-1 antibody can significantly enhance the therapeutic efficacy of CAR T-cell therapy. The combination of these two modalities is likely to have a significant impact on increasing the effectiveness of immunotherapy against a number of cancers that are currently resistant to first-line treatment.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors' Contributions
Conception and design: L.B. John, M.H. Kershaw, P.K. Darcy Development of methodology: L.B. John, M.H. Kershaw, P.K. Darcy Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): L.B. John, C. Devaud, C.M. Duong, C. Yong, P.A. Beavis, N.M. Haynes, P.K. Darcy Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): L.B. John, C. Devaud, M.H. Kershaw, P.K. Darcy Writing, review, and/or revision of the manuscript: L.B. John, C.M. Duong, C. Yong, P.A. Beavis, N.M. Haynes, M.T. Chow, M.J. Smyth, P.K. Darcy Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): L.B. John, M.T. Chow, P.K. Darcy Study supervision: M.J. Smyth, M.H. Kershaw, P.K. Darcy

Acknowledgments
The authors thank the Peter MacCallum Cancer Centre Experimental Animal Facility technicians for the assisting in animal care and the Histology Department for processing of H&E sections of tissue following therapy.

Grant Support
This work was funded by Project Grants from the National Health and Medical Research Council (NHMRC), Cancer Council of Victoria, and the Susan Komen Breast Cancer Foundation. M.J. Smyth was supported by an NHMRC Australia Research Fellowship. M.H. Kershaw and P.K. Darcy were supported by NHMRC Senior Research Fellowships.

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Received February 20, 2013; revised May 15, 2013; accepted July 4, 2013; published OnlineFirst July 19, 2013.

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Clin Cancer Res  Published OnlineFirst July 19, 2013.

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doi:10.1158/1078-0432.CCR-13-0458

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